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ORIGINAL PAPER

Single-pot derivatisation strategy for enhanced gliotoxin detection by HPLC and MALDI-ToF mass spectrometry

Carol Davis · Natasha Gordon · Sinéad Murphy · Ishwar Singh · Kevin Kavanagh · Stephen Carberry · Sean Doyle

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Abstract Gliotoxin is produced by non-ribosomal peptide synthesis and secreted from certain fungi, including *Aspergillus fumigatus*. It is an epipolythiodioxopiperazine that contains an intact disulphide bridge and is the focus of intense research as a consequence of its negative immunomodulatory properties. Gliotoxin detection is generally enabled by reversed-phase–high-performance liquid chromatography (RP-HPLC), with absorbance detection (220–280 nm), or liquid chromatography-mass spectrometry, yet detection is not readily achievable by matrix-assisted laser desorption ionisation–time-of-flight mass spectrometry (MALDI-ToF MS). We have developed a single-pot derivatisation strategy which uses sodium borohydride-mediated reduction of gliotoxin followed by immediate alkylation of exposed thiols by 5′-iodoacetamidofluorescein to yield a stable product, diacetamidofluorescein-gliotoxin (GT-$(AF)_2$), of molecular mass 1103.931 Da ((M+H)+). This product is readily detectable by RP-HPLC and exhibits a 6.8-fold increase in molar absorptivity compared with gliotoxin, which results in a higher sensitivity of detection (40 ng; 125 pmoL). GT-$(AF)_2$ also fluoresces (excitation/emission, 492:518 nm). Unlike free gliotoxin, the product (>800 fmol) is detectable by MALDI-ToF MS. Sporidesmin A can also be detected by RP-HPLC and MALDI-ToF MS (>530 fmol) using this strategy. We also demonstrate that the strategy facilitates detection of gliotoxin (mean± SD=3.55±0.07 μg 100 μL^{-1}; n=2) produced by *A. fumigatus*, without the requirement for organic extraction of culture supernatants and associated solvent removal. GT-$(AF)_2$ is also detectable (150 ng; 460 pmol) by thin-layer chromatography.

Keywords *Aspergillus fumigatus* · Epipolythiodioxopiperazine · Gliotoxin · NRPS · Redox · Sporidesmin A.

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Introduction

Gliotoxin is secreted by *Aspergillus fumigatus* and other fungi when grown in culture or during infection of a host organism, where is can exert immunosuppressive effects (Fig. 1) [1, 2]. It is an epipolythiodioxopiperazine (ETP)-type toxin (326 Da) which contains a disulphide bridge of unknown origin. This structural feature of gliotoxin plays a highly significant role in mediating the virulence of *A. fumigatus* [3, 4]. Gliotoxin is not immunogenic, and its toxicity in mammalian cells is generally enabled by direct inactivation of essential protein thiols, inhibition of NADPH oxidase assembly, in addition to promotion of redox cycling which leads to hydrogen peroxide formation [1, 5–7]. Sporidesmin A (Fig. 1) is also an ETP-type metabolite which contains a disulphide bridge and is

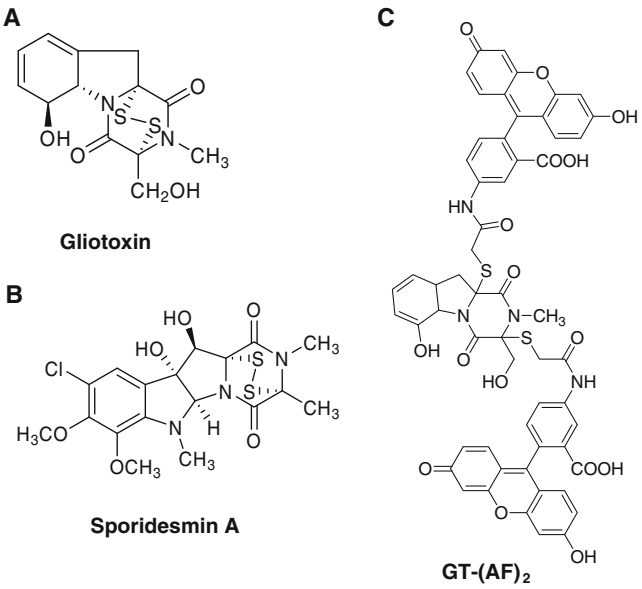

Fig. 1 **A** Gliotoxin, **B** sporidesmin A, and **C** proposed structure of diacetamidofluorescein-gliotoxin (GT-$(AF)_2$)

produced by the animal disease-causing fungus, *Pithomyces chartarum* [3].

The detection of fungal-specific peptide biomarkers such as gliotoxin represents an emerging strategy for the reliable diagnosis of fungal infection which, to date, has proved to be extremely difficult [8, 9]. Gliotoxin detection is normally by reversed-phase high-performance–liquid chromatography (RP-HPLC) with UV absorbance detection (220–280 nm), gas chromatography-mass spectrometry or liquid chromatography-mass spectrometry (LC-MS) analysis [10–13]. More specifically, gliotoxin detection following RP-HPLC separation is usually carried out at 254 nm [10]. The immunological detection of gliotoxin by ELISA has been demonstrated, and here, the limit of detection was 10 μg mL^{-1} [14]. In addition, Lewis et al. showed that gliotoxin was present in sera and lungs of mice suffering from experimentally induced invasive aspergillosis by LC-MS analysis [15]. These authors also demonstrated that gliotoxin could be detected, by LC-MS, in cancer patients with either proven or probable invasive aspergillosis [15].

Studies on gliotoxin biosynthesis, which have demonstrated the role of the non-ribosomal peptide synthetase, GliP, in mediating the initial step of gliotoxin formation, have also deployed LC-MS to detect the presence and absence of gliotoxin in wild-type and mutant (Δ*gliP*) cultures from *A. fumigatus* [16]. Moreover, using a chemical reduction and alkylation strategy, thiol and disulphide absence has been confirmed in a gliotoxin-like metabolite isolated from an *A. fumigatus* mutant deficient in gliotoxin biosynthesis [17]. To date, matrix-assisted laser desorption ionisation–time-of-flight mass spectrometry (MALDI-ToF MS) has not been used for the detection of gliotoxin, as matrix components can interfere with the detection of low molecular mass metabolites and gliotoxin fragments during ionisation, thereby rendering detection impossible [16, 18].

The constituent disulphide bridge of gliotoxin can be cleaved and converted to two thiol groups by reduction with compounds such as Cleland's reagent/dithiothreitol or β-mercaptoethanol [19]. However, these low molecular mass molecules cannot be removed by dialysis or gel filtration from reduced gliotoxin, and their presence will interfere with any subsequent chemical modification (e.g., alkylation) of exposed thiols. It has also been demonstrated that glutathione-mediated reduction of gliotoxin is possible but results in the formation of mixed disulphides [20, 21] which would be refractory to subsequent modification through blocked thiols. Alternative reducing agents compatible with post-reduction labelling are therefore required, and Woodcock et al. have demonstrated that sodium borohydride ($NaBH_4$) can reduce both gliotoxin and sporidesmin A [22].

In the present work, $NaBH_4$ was used to reduce the disulphide bridge of gliotoxin to generate thiol groups which could react with 5′-iodoacetamidofluorescein (5′-IAF) to yield a stable derivative. This reaction product is detectable at higher sensitivity than unlabelled gliotoxin, by RP-HPLC, and could also be detected by MALDI-ToF MS and thin-layer chromatography analysis (TLC).

Materials and methods

Materials and reagents

All reagents were obtained from Sigma-Aldrich, Dorset, UK, unless otherwise indicated. 5′-IAF was prepared in dimethyl sulfoxide (DMSO) at 3–10 mg mL^{-1}. $NaBH_4$ (500 mM) was prepared in HPLC-grade water and used within 30 min of preparation. *A. fumigatus* strains Af293 (wild-type) and Δ*gliZ* (a kind gift from Professor Nancy Keller, University of Wisconsin-Madison), a binuclear cluster domain transcription factor-deficient mutant incapable of gliotoxin biosynthesis [23], were cultured as described elsewhere [17, 24]. Sporidesmin A was purchased from AgResearch (Hamilton, New Zealand).

Reduction and alkylation of gliotoxin under organic or aqueous conditions

Solutions containing gliotoxin (100 μL; 100 μg mL^{-1} methanol; 30.6 nmoL gliotoxin) were prepared. To these were added 2.5 μL 500 mM $NaBH_4$ (1.2 μmol $NaBH_4$), followed by gentle mixing by vortexing and incubation for 60 min at room temperature (occasionally 50 mM $NaBH_4$

was used as reductant, as indicated). Labelled sporidesmin A was prepared under identical conditions. No $NaBH_4$ was added when the intact disulphide bridge of gliotoxin was required (i.e. as negative control), and gliotoxin preparations were also independently reduced using 6 μL dithiothreitol (DTT; 10 mM in deionised water), which contains free thiol groups, for comparison with $NaBH_4$ as reducing agent. 5′-IAF (20 μL, 3 mg mL^{-1}; 120 nmol) was added to reduced or oxidised gliotoxin preparations (30.6 nmol), followed by vortexing of the resultant mixtures briefly and incubation for 40 min in the dark at room temperature prior to HPLC analysis. Alkylation of reduced gliotoxin was also undertaken as described above using excess iodoacetamide (400 nmol) instead of 5′-IAF. Tris(2-carboxyethyl) phosphine hydrochloride (TCEP·HCl; Thermo Scientific) could also be used as reducing agent (125 nmol per reaction) prior to 5′-IAF-mediated alkylation.

RP-HPLC analysis of culture supernatants (48 h; adjusted to pH 7.5 using 0.1 volume 1 M Na_2HPO_4 pH 7.5) of *A. fumigatus* Af293, spiked with gliotoxin and following $NaBH_4$ (500 mM) reduction and subsequent labelling with 5′-IAF was also undertaken. Here, gliotoxin (327 μg mL^{-1}; 50 μL) was added to 50 μL *A. fumigatus* culture supernatants pH 7.5, followed by addition of 50 μL dimethyl sulfoxide. Sodium borohydride (3.3 μL; 50 or 500 mM) was then added followed by 10 μL 5′-IAF (10 mg mL^{-1}). Modification of endogenous gliotoxin in culture supernatants, under aqueous conditions, via sodium borohydride reduction and 5′-IAF labelling was undertaken as follows—2.5 μL 500 mM $NaBH_4$ and 20 μL 5′-IAF (3 mg mL^{-1}; 120 nmol) were added per 100 μL test specimen (*A. fumigatus* culture supernatants, pH 7.5) and incubated sequentially as indicated above. Endogenous gliotoxin was also chloroform-extracted from *A. fumigatus* culture supernatants and resuspended in methanol [17]. Extensive assay validation and investigation included evaluation of alternative chromatographic conditions to detect labelled gliotoxin, compatibility with MALDI-ToF MS, sensitivity of detection, reproducibility, reductive alkylation of gliotoxin under aqueous conditions, sporidesmin A compatibility and TLC detection.

RP-HPLC analysis

Reversed-phase HPLC used solvent A 0.1% (*v/v*) trifluoroacetic acid in HPLC-grade water and solvent B 0.1% (*v/v*) trifluoroacetic acid in 100% (*v/v*) acetonitrile. Chromatography, using a C_{18} column (Agilent Zorbax Eclipse XDB-C18; 5-μm particle size; 4.6×15 mm) was carried out using an Agilent Series 1,200 HPLC system equipped with diode array (DAD) and fluorescence detectors. Gradient HPLC conditions (three) were as follows: method 1, 35–60% solvent B over 6 min (Δ%B/min=4.0); method 2, 5–100% solvent B over 20 min (Δ%B/min=4.75) or method 3, over 24 min (Δ%B/min=4.0) and were employed for gliotoxin, reduced gliotoxin, and labelled gliotoxin separation followed by absorbance detection (220 or 254 nm) plus fluorescence detection (excitation, 492 nm; emission, 518 nm). Isocratic conditions were always established prior to specimen injection and the column washed and re-equilibrated prior to subsequent specimen injection. Sporidesmin A was similarly analysed. Specimen injection volume was 20 μL, unless otherwise noted, at a solvent flow rate of 1 mL min^{-1}.

MALDI-ToF MS

Mass spectrometry was carried out using an Ettan™ MALDI-ToF mass spectrometer (Amersham Biosciences (Europe) GmbH, Freiburg, Germany). Specimens (0.5 μL), neat or diluted up to 1/200 in 0.1% (*v/v*) trifluoroacetic acid, for mass determination were layered upon 0.5 μL α-cyano-4-hydroxycinnamic acid (5 mg 200 μL^{-1} 50%(*v/v*) acetonitrile in 0.1% (*v/v*) aqueous trifluoroacetic acid), previously deposited onto mass spectrometry slides and allowed to dry prior to delayed extraction, reflectron ToF analysis at 20 kV [24]. Internal calibrants, angiotensin III and adrenocorticotropic hormone fragments 18–39, were used to calibrate spectra.

Thin-layer chromatography analysis

TLC analysis of reduced and labelled gliotoxin, 5′-IAF only, and unreduced gliotoxin in the presence of 5′-IAF was performed. TLC analysis used Merck silica gel 60-F254 TLC plates (aluminium backed). Solvent systems were dichloromethane/methanol (90:10; containing 100 μL acetic acid per 10 mL) or dichloromethane/methanol (94:6). The sample application volume was 1 μL. Images were obtained using a Fluorescence Scanner (Typhoon Variable Mode Imager; GE Healthcare) at excitation and emission wavelengths at 488 and 520 nm, respectively (sensitivity setting, 600 V; 50 μm pixel size).

Results

Alkylation of $NaBH_4$-reduced gliotoxin enhances detection

$NaBH_4$-mediated reduction enables the subsequent alkylation of exposed thiols on gliotoxin using 5′-IAF; moreover, removal of the reducing agent is not required prior to alkylation. Figure 2a illustrates the formation of 5′-IAF-labelled gliotoxin (retention time (R_T)=5.18 min), following RP-HPLC separation and detection at 254 nm. Unreacted 5′-IAF is also visible upon chromatography (R_T=4.66 min). Labelled gliotoxin is not present when the disulphide bridge of gliotoxin remains intact (in the absence of $NaBH_4$) and

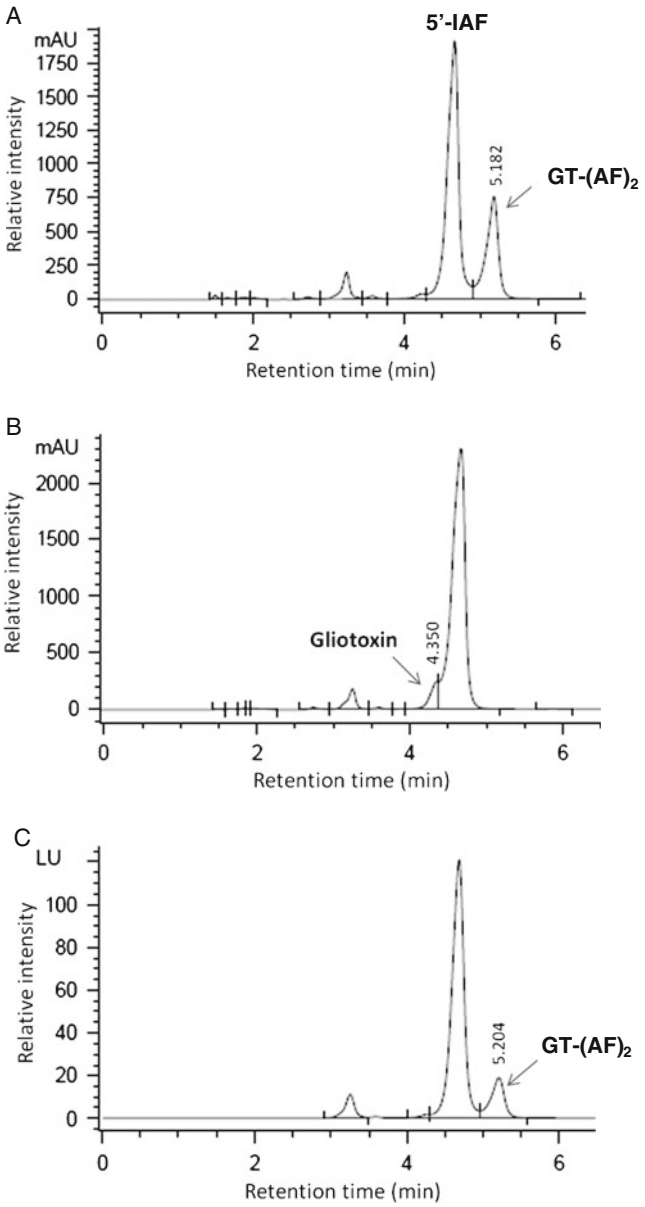

Fig. 2 RP-HPLC analysis of pure gliotoxin (Sigma-Aldrich) with **A** and without **B** sodium borohydride-mediated reduction prior to 5′-IAF labelling. **A** Gliotoxin+$NaBH_4$+5′-IAF: Gliotoxin disulphide bridge is reduced and SH groups (thiols) alkylated by 5′-IAF. The fluorescently labelled form of gliotoxin (GT-$(AF)_2$) was detectable at 254 nm with a retention time of 5.18 min as indicated in (**A**). **B** Gliotoxin disulphide intact: No fluorescent labelling of gliotoxin possible. Free gliotoxin (GT) is detectable at 254 nm with a retention time of 4.35 min. Identical amounts of gliotoxin were present in each assay, yet the labelled form exhibits higher absorbance, compared with free gliotoxin, at 254 nm. **C** Fluorescence detection of labeled gliotoxin following HPLC separation. Excitation and emission 492/518 nm. Fluorescently labelled gliotoxin is evident at retention time of 5.20 min (slightly later than that for a 254 nm detection (5.18 min) as the fluorescence detector was in series after DAD)

therefore unavailable for alkylation (Fig. 2b). However, free gliotoxin is evident in Fig. 2b (R_T=4.35 min). Although the amounts (10 μg) of gliotoxin initially present in reaction mixtures were identical, absorbance of 5′-IAF labelled gliotoxin (peak area (mean±SD)=8,772±606; n=3) was enhanced relative to the unlabelled reduced or oxidised form (peak area (mean±SD)=1,287±122; n=3). This observed increase in A_{254nm} of labelled gliotoxin is clearly due to an increase in the molar extinction coefficient (ε) relative to free gliotoxin and indicates a 6.8-fold increase in sensitivity of detection for labelled, compared with free, gliotoxin detection. The labelled form of gliotoxin also fluoresces, although it appears that the quantum yield of the fluorescein component is suppressed at the low pH conditions employed for RP-HPLC (Fig. 2c). Here, fluorescently labelled gliotoxin is detected at R_T=5.20 min, and no fluorescently labelled gliotoxin is evident without prior reduction (data not shown). Thus, identification of labelled gliotoxin at 254 nm enhances the specificity, as well as sensitivity, of the method over pre-existing strategies for direct gliotoxin detection.

Modified gliotoxin is detectable by MALDI-ToF MS

Detection of gliotoxin by MALDI-ToF MS is confounded by both small molecular size and ionisation-induced fragmentation, however, alkylation of gliotoxin results in a stable molecular species readily detectable by MALDI-ToF MS. Both thiol groups of reduced gliotoxin were covalently alkylated by 5′-IAF to form a diacetamidofluorescein derivative of gliotoxin, confirmed by the appearance of a species of *m/z* 1103.931 ($(M+H)^+$) which corresponds precisely to the protonated form of doubly acetamidofluorescein-labelled gliotoxin (i.e. GT-$(AF)_2$) (1102.93 Da), as determined by MALDI-ToF MS analysis (Fig. 3). The proposed molecular structure of GT-$(AF)_2$ is shown in Fig. 1c. Reduction and 5′-IAF treatment of a gliotoxin-deficient strain (*A. fumigatus* Δ*gliZ* [23]) did not yield any GT-$(AF)_2$, thereby confirming the specificity of gliotoxin detection using this strategy (data not shown). No product was detectable by MALDI-ToF MS either in the absence of prior reduction or the absence of 5′-IAF (data not shown). Gliotoxin was also labelled by iodoacetamide-mediated alkylation, resulting in complete disappearance of oxidised gliotoxin from solution; however, no evidence of absorbance enhancement of alkylated gliotoxin was apparent in this case (data not shown). Neither was any molecular species of *m/z* 384.96 or 443.02, which would correspond to the mass of mono- or diacetamido-gliotoxin, respectively, detectable by MALDI-ToF MS. Thus, it appears that only 5′-IAF, and not iodoacetamide, is a suitable agent to stabilise gliotoxin for subsequent MALDI analysis.

Derivatised sporidesmin A detection by RP-HPLC and MALDI-ToF MS

Sporidesmin A underwent $NaBH_4$-mediated reduction and was also subjected to alkylation. RP-HPLC analysis using

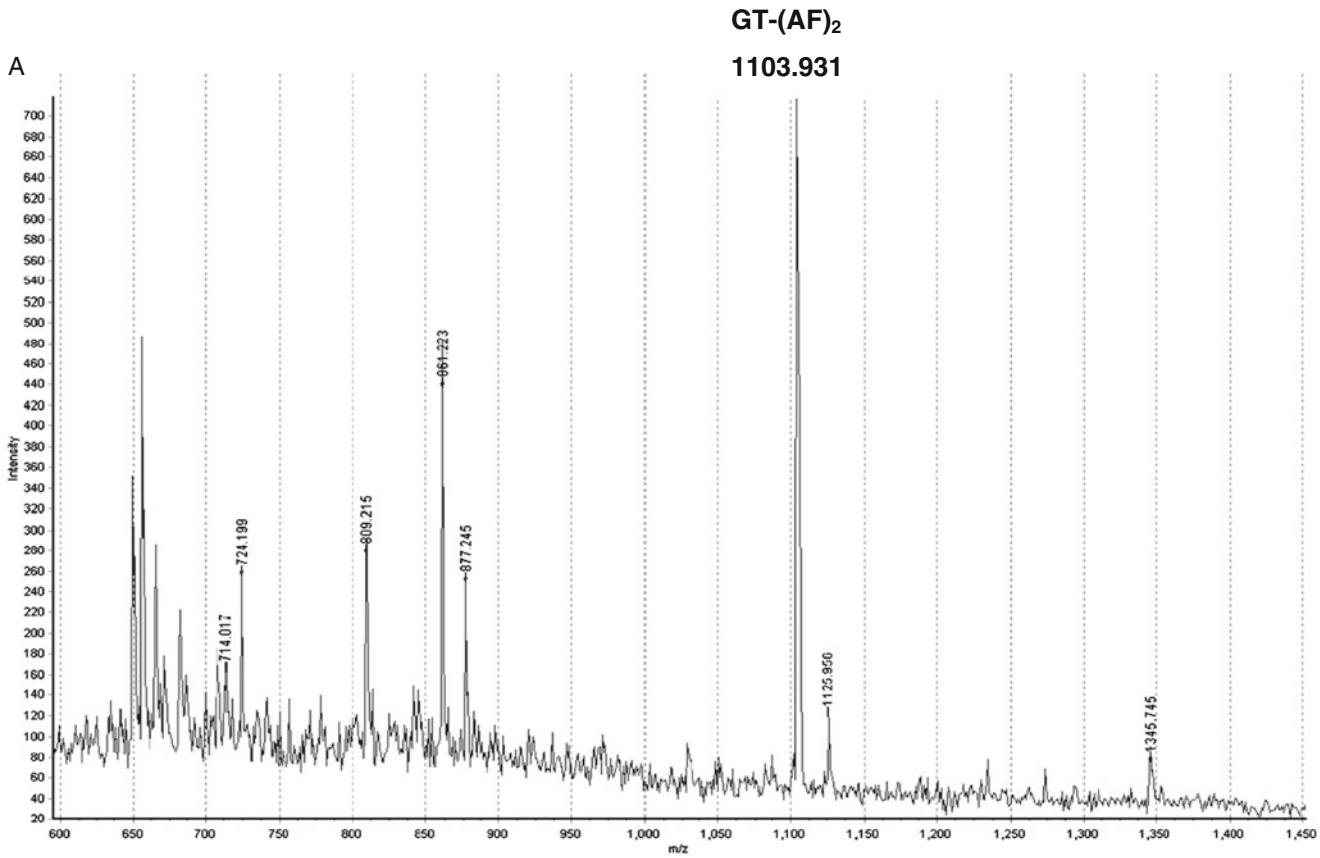

Fig. 3 MALDI-ToF mass spectrum of GT-$(AF)_2$ (800 fmol) prepared by reduction and alkylation of gliotoxin. A molecular species of *m/z* 1103.931 ((M+H)+) is detectable which corresponds precisely to protonated diacetamidofluorescein–gliotoxin. No product was detectable either in the absence of prior reduction or the absence of 5′-IAF

method 1 demonstrated that reduction resulted in a shift in retention time from 7.27 to 5.48 min (Fig. 4a, b) and that sporidesmin A could be derivatised with 5′-IAF (only after reduction) to yield a product, sporidesmin A-$(AF)_2$, which eluted at 7.96 min (Fig. 4c, d). Sporidesmin A-$(AF)_2$ (>530 fmol) was detectable by MALDI-ToF MS, as described for GT-$(AF)_2$, as a charged species at *m/z* 1251.145, which corresponds precisely to the predicted protonated mass of the derivative (Fig. 4e). No sporidesmin A-$(AF)_2$ was detectable without the presence of $NaBH_4$ in the reaction mixtures (data not shown).

$NaBH_4$ is the preferred reductant for gliotoxin

Data in Fig. S1 (Electronic Supplementary Material) illustrate that GT-$(AF)_2$ is detectable at 254 nm with a retention time of 15.24 min using either DTT (peak area (mean±SD)=1,833±1,543; n=3) (Fig. S1a, Electronic Supplementary Material) or $NaBH_4$ (peak area (mean± SD)=8,772±606; n=3) (Fig. S1b, Electronic Supplementary Material) as reducing agent. However, it is clear that the use of DTT as reductant results in diminished formation of labelled gliotoxin (fivefold lower), most likely due to a reduced rate of disulphide bridge cleavage relative to $NaBH_4$ and competition with gliotoxin-thiols for reaction with 5′-IAF. Moreover, residual oxidised gliotoxin (R_T= 14.42 min) was present (Fig. S1a, Electronic Supplementary Material) indicating that DTT is an inferior reductant compared with $NaBH_4$ for cleavage of the disulphide bridge on gliotoxin. $NaBH_4$-mediated reduction of gliotoxin is complete within a maximum of 15 min (mean± SD=1.5±0.23 μg; n=3), and reduced gliotoxin does not exhibit any evidence of re-oxidation for up to 2 h post-reduction (mean±SD=1.5+0.11 μg; n=3). TCEP addition also resulted in formation of reduced gliotoxin which was subsequently alkylated with 5′-IAF (data not shown); however, use of TCEP required reaction pH 7.6 when gliotoxin modification was undertaken in organic solvents. No pH adjustment was necessary using $NaBH_4$ under these conditions.

Assay validation

A calibration curve for gliotoxin detection using sequential reduction and labelling is shown in Fig. 5, and a linear response is observed between 0 and

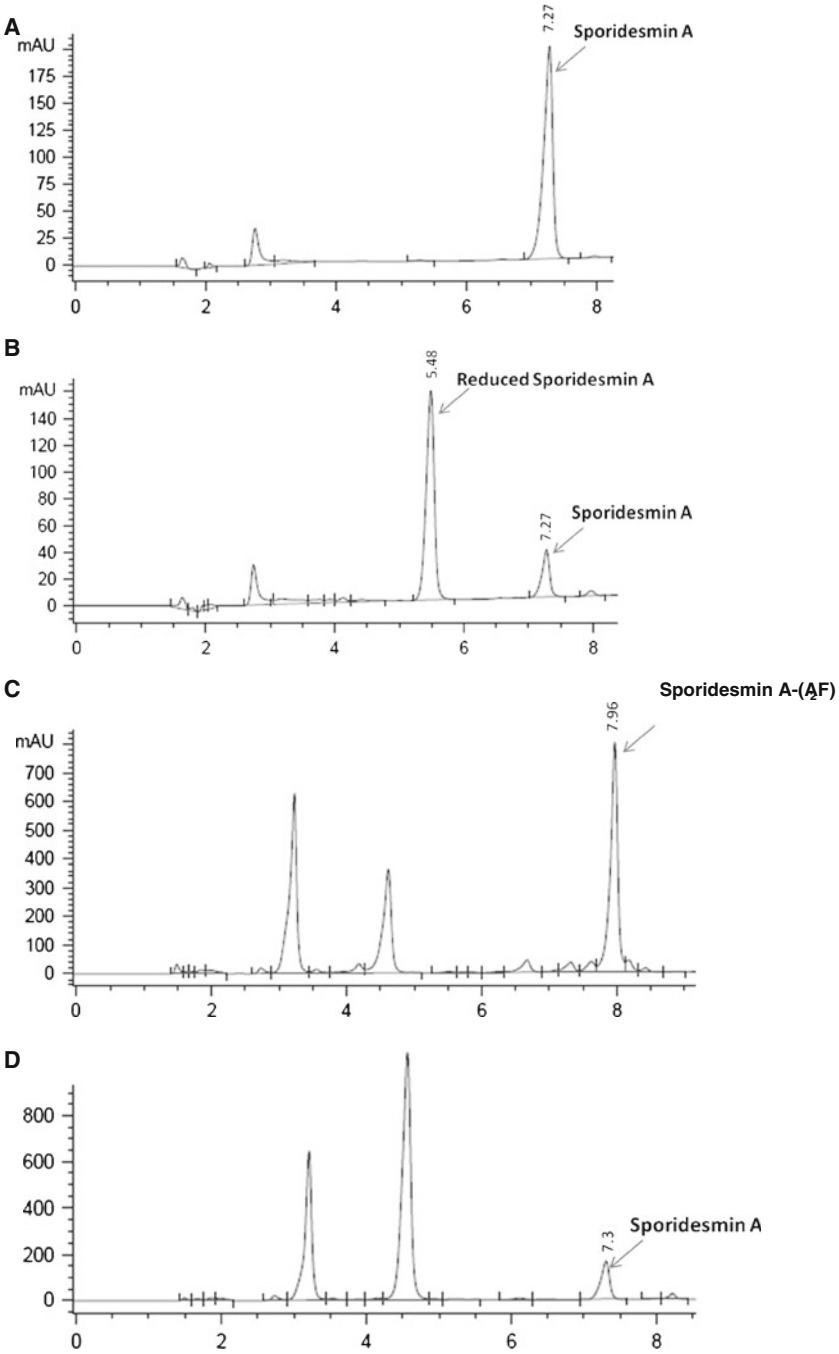

Fig. 4 RP-HPLC analysis of pure sporidesmin A **A** prior to and **B** post-sodium borohydride-mediated reduction prior to 5′-IAF labelling. **C** Sporidesmin A+$NaBH_4$+5′-IAF: disulphide bridge is reduced and SH groups (thiols) alkylated by 5′-IAF. Sporidesmin A-$(AF)_2$ was detectable at 254 nm with a retention time of 7.96 min. **D** Sporidesmin A+5′-IAF: only free sporidesmin A is present (7.3 min). Without reduction, sporidesmin A-$(AF)_2$ is absent. **E** MALDI-ToF mass spectrum of sporidesmin A-$(AF)_2$ prepared by reduction and alkylation (530 fmol). A molecular species of *m/z* 1251.145 ((M+H)+) is detectable which corresponds precisely to protonated diacetamidofluorescein–sporidesmin A. No product was detectable either in the absence of prior reduction or the absence of 5′-IAF (data not shown)

2,000 pmoL gliotoxin, following RP-HPLC at 254 nm. The limit of detection for free gliotoxin is 125 pmoL (40 ng). Intra-assay reproducibility (n=5) was determined to range between 13% and 14% CV, and interassay reproducibility (n=5) was calculated to be between 13% and 16% CV, between for 10–30 nmol gliotoxin (Table S1). GT-$(AF)_2$ exhibited a mean post-synthesis stability of 88% during storage at 20–25 °C for 24 h in the dark. Thus, for long-term storage (up to 1 month) of GT-$(AF)_2$, −20 °C is recommended.

Reduction and alkylation facilitates detection of gliotoxin produced by *A. fumigatus*

To further confirm the utility of the method for gliotoxin detection, organic extracts from 48-h cultures of *A. fumigatus* Af293 were prepared and subjected to reduction and alkylation with 5′-IAF (Fig. S2, Electronic Supplementary Material). Although GT-$(AF)_2$ exhibits enhanced absorbance at 220 versus 254 nm, the majority of analyses were performed at the latter wavelength to minimise solvent

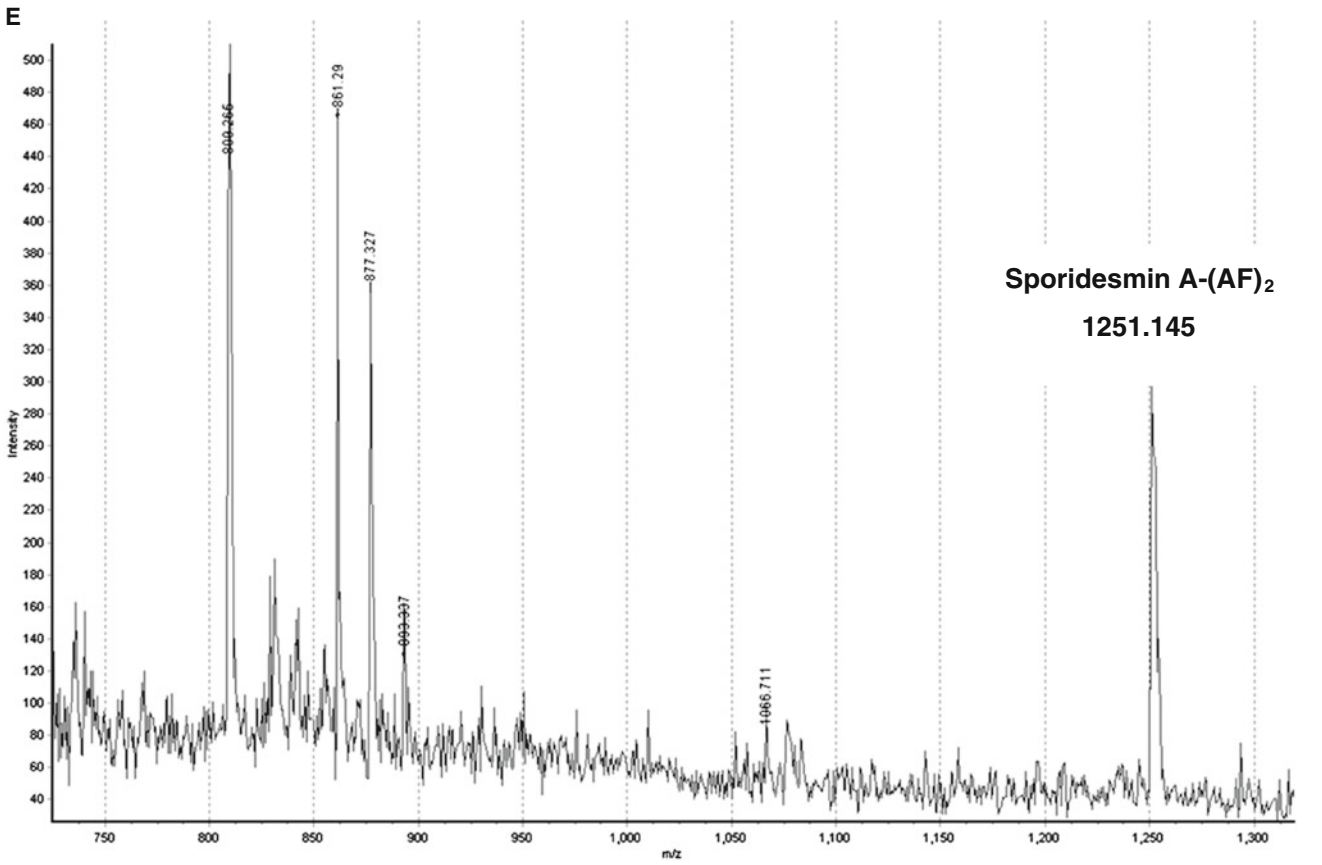

Fig. 4 (continued)

absorbance effects and yield clearer chromatograms. Data presented in Fig. 6a illustrate that spiked gliotoxin can also be detected in *A. fumigatus* culture supernatants without the need for prior extraction. This strategy required the upward pH adjustment of culture supernatants (final pH 7.5) and addition of DMSO to ensure solubility of 5′-IAF during the labelling. Interestingly, it was noted that use of stock solutions of $NaBH_4$ (500 mM as opposed to 50 mM) specifically suppressed the absorbance of unreacted 5′-IAF while that of GT-$(AF)_2$ was unaffected (Fig. 6b). Detection and quantitation of native gliotoxin in *A. fumigatus* culture supernatants (mean ±SD=3.55±0.07 μg/100 μL; n=2), without the need for prior organic extraction, and organic solvent (DMSO) addition to reaction mixtures, was also achieved (Fig. 6c). Addition of lower amounts of 5′-IAF, as a consequence of lower amounts of endogenous gliotoxin, obviated the requirement to add DMSO to maintain 5′-IAF solubility. Labelled gliotoxin was absent without prior reduction (Fig. 6d).

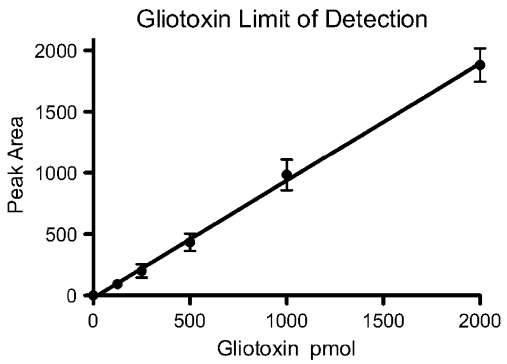

Fig. 5 Calibration curve for gliotoxin detection by sequential reduction and alkylation (0–2,000 pmoL gliotoxin). Assay conditions are described in "Materials and methods", and *error bars* represent mean amount±SD (n=3)

Thin-layer chromatography

Finally, a TLC method for the detection of labelled gliotoxin has also been developed (Fig. S3, Electronic Supplementary Material). Solvent optimisation was necessary in order to achieve the required resolution between GT-$(AF)_2$ and 5′-IAF (data not shown). The optimal method utilised Merck silica gel 60-F254 TLC plates (aluminium backed), a dichloromethane/methanol/acetic acid (90:10:1) solvent system, can be completed in 30 min and represents a strategy for high-throughput, simultaneous analysis of multiple test specimens. Detection of 150 ng (460 pmoL) GT-$(AF)_2$ was evident, by fluorescence scanning (sensitivity setting, 600 V), only when

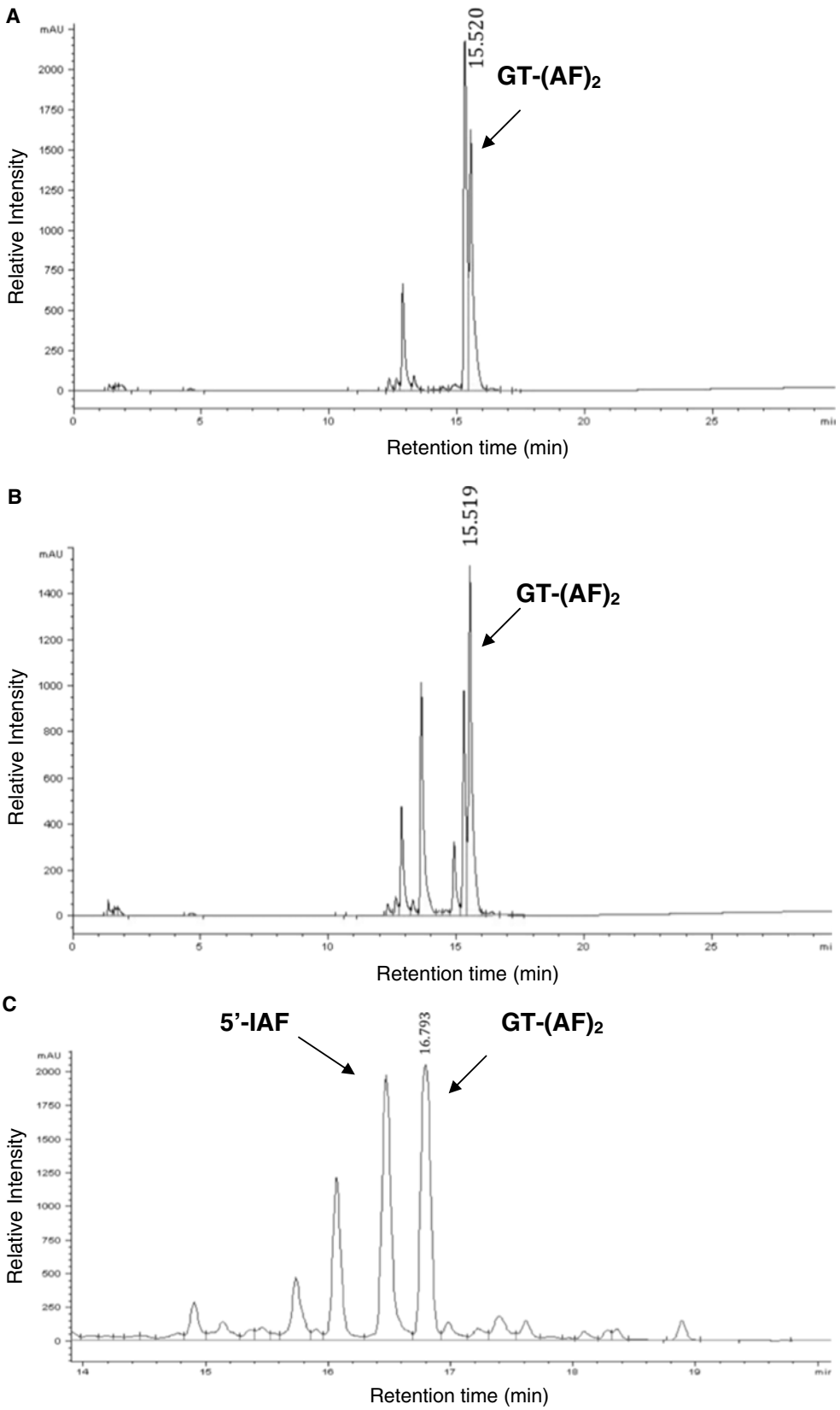

Fig. 6 Optimised RP-HPLC detection of GT-$(AF)_2$ prepared under aqueous conditions. **A** RP-HPLC analysis (method 2) of culture supernatant (48 h; adjusted to pH 7.5) of *A. fumigatus* Af293 strain, spiked with gliotoxin and following $NaBH_4$ (50 mM) reduction and subsequent labelling with 5′-IAF. The presence of GT-$(AF)_2$ was observed at 15.52 min. **B** RP-HPLC analysis of culture supernatants (48 h; adjusted to pH 7.5) of *A. fumigatus* Af293, spiked with gliotoxin and following $NaBH_4$ (500 mM) reduction and subsequent labelling with 5′-IAF. Gliotoxin (327 μg mL^{-1}; 50 μL) was added to 50 μL *A. fumigatus* culture supernatant pH 7.5 followed by addition of 50 μL dimethyl sulfoxide. Sodium borohydride (3.3 μL; 50 or 500 mM) was then added followed by 10 μL 5′-IAF (10 mg mL^{-1}). HPLC injection volume was 20 μL (equivalent to 2 μg gliotoxin). **C** RP-HPLC analysis (method 3) of culture supernatants (48 h; adjusted to pH 7.5) of *A. fumigatus* Af293 followed reduction and subsequent labelling with 5′-IAF. The presence of GT-$(AF)_2$ (R_T, 16.79 min) was observed. **D** RP-HPLC analysis, using method 3, of culture supernatant (48 h; adjusted to pH 7.5) following addition of 5′-IAF (20 μL; 3 mg mL^{-1}; 120 nmol), without prior reduction. No GT-$(AF)_2$ was evident. Free gliotoxin was observed (R_T, 15.49 min). Absorbance detection at 254 nm

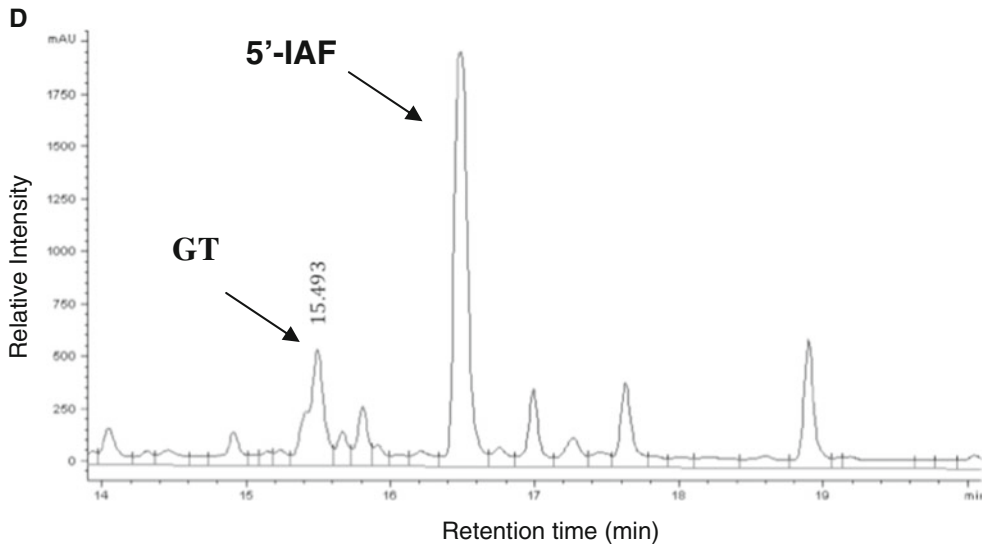

Fig. 6 (continued)

labelling has been preceded by $NaBH_4$ reduction (Fig. S3, Electronic Supplementary Material).

Discussion

Improved methods for the sensitive and specific detection of gliotoxin have been developed. Firstly, it has been shown that the reducing agent, $NaBH_4$, does not have to be removed prior to subsequent alkylation of gliotoxin, or sporidesmin A, thiols using 5′-iodoacetamidofluorescein (5′-IAF). Secondly, it appears that the resultant diacetamidofluorescein derivative of gliotoxin (GT-$(AF)_2$) has a higher molar absorption (6.8-fold) than unlabelled gliotoxin and so, should facilitate improved detection. Unlike free gliotoxin, the modified form can be detected by MALDI-ToF MS and has been shown to exhibit a molecular mass of 1103.93 Da. Our results also confirm that *A. fumigatus*-derived gliotoxin in organic extracts can be identified and that added- and native-gliotoxin is detectable in pH-adjusted culture supernatants. In addition, it appears that $NaBH_4$ is a superior gliotoxin-reducing agent than DTT and that increased amounts of $NaBH_4$ during modification steps may suppress unreacted 5′-IAF interference during subsequent RP-HPLC analysis. Finally, we demonstrate that labelled gliotoxin can also be detected by TLC.

Conventional RP-HPLC methods, which use UV absorbance for detecting gliotoxin, depend solely on the R_T of gliotoxin in test specimens compared with that of a standard preparation [10, 20]. While this approach is acceptable, the number of fungal metabolites which can be present in organic extracts, combined with the possibility of peptide generation due to unwanted proteolysis, demands a more rigorous confirmation of gliotoxin presence. LC-MS analysis of gliotoxin fulfils this criterion; however, this is a relatively specialised technique and is not available to all researchers [12, 15, 16]. Moreover, alternative bioassay formats have also been proposed for detection of gliotoxin and related epipolythiodioxopiperazines, of fungal origin, at levels of 18–20 ng $well^{-1}$ [19, 25]. Although the sensitivity of the Microtox® test is in the low micrograms-per-milliliter-range [25], the specificity of such tests, which deploy the sensitivity of bacterial luminescence to mycotoxin presence, as a measure of gliotoxin presence, is unclear. Sequential reduction and alkylation of gliotoxin, followed by detection using RP-HPLC, MALDI-ToF MS, or TLC represents a new direction for the improved detection of gliotoxin, whereby the molar absorption of the labelled form is almost sevenfold greater than free gliotoxin. This observation of enhanced sensitivity has not been forthcoming from elegant investigations of glutathione detection following modification with *N*-(1-pyrenyl)maleimide and subsequent HPLC identification of the resultant product [26]. Although GT-$(AF)_2$ was fluorescent, it was observed that fluorescence quenching, under the acidic conditions employed for RP-HPLC identification, was apparent. Indeed, it has previously been noted that the quantum yield of fluorescein is significantly quenched in low pH environments [27] which further emphasises the practical significance of the enhanced absorbance of GT-$(AF)_2$ at 220–254 nm.

$NaBH_4$ reduction of protein disulphides prior to reaction with 4,4′-dithiodipyridine (Aldrithiol-4®) and subsequent thiol quantitation by detection of liberated 4-thiopyridone ($A_{324\ nm}$) has been proposed to enable picomole detection of total protein thiols [28]. However, this approach required high temperature incubation (50 °C), addition of hexanol to prevent foaming during reduction, and destruction of residual $NaBH_4$ by acidification, prior to 4, 4′-dithiodipyridine addition. The use of $NaBH_4$ for gliotoxin reduction, which occurs instantaneously at room temperature (20 °C) requires none of these precautions. In fact, our results indicate that additional $NaBH_4$ (tenfold) may actually assist the detection of the di-acetamidofluorescein derivative of gliotoxin by degrading free 5′-IAF present after reaction. In addition, the direct detection of the labelled gliotoxin product confers a degree of specificity not associated with 4-thiopyridone release which is an indirect measure of thiol presence and could, in theory, result from non-specific reduction of 4, 4′-dithiodipyridine.

The occurrence of ETP-encoding gene clusters and studies directed towards elucidation of gliotoxin biosynthesis, and toxicity, are the focus of significant current effort [1, 2, 23, 29–33]. Thus, the development of a methodology for the specific detection of gliotoxin, especially under aqueous conditions and without the requirement for prior organic extraction and solvent removal, is significant because it enables direct identification of gliotoxin without specimen pre-treatment which may otherwise lead to gliotoxin loss or modification. Moreover, as with alternative strategies for mycotoxin detection [34], the reductive alkylation approach developed for detection of gliotoxin will also be useful in the detection of related ETP toxins such as sporidesmin A, as we have demonstrated, and other disulphide- or thiol-containing molecules [35, 36].

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